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Research article

Growth and organotypic branching of lung-specific microvascular cells on 2D and in 3D lung-derived matrices

Sena Nur Ozkan[1,2], Ece Ozturk[*1,2,3]

[1] *Koc University, Engineered Cancer and Organ Models Laboratory, 34450, Istanbul, Türkiye*
[2] *Koc University, Research Center for Translational Medicine (KUTTAM), 34450, Istanbul, Türkiye*
[3] *Koc University, Department of Medical Biology, School of Medicine, 34450, Istanbul, Türkiye*

Abstract

Tissue-specific endothelial cells have vital roles in maintenance and functioning of native tissues with constant reciprocal crosstalk with resident cells. Three-dimensional (3D) physio-mimetic *in vitro* models which incorporate lung-specific microvasculature are needed to model lung-related diseases which involve modulation of endothelial cell behavior like cancer. In this study, we investigated the growth kinetics, morphological changes and responses to biological cues of lung microvasculature on two-dimensional (2D) and in lung matrix-derived 3D hydrogels. HUVEC and HULEC-5a cells were cultured on 2D and compared for their growth, morphologies, and responses to varying growth medium formulations. Brightfield and immunofluorescence imaging was performed to assess differences in morphology. For 3D cultures, native bovine lungs were decellularized, lyophilized, solubilized, and reconstituted into hydrogel form in which endothelial cells were embedded. Cell growth and organotypic branching was monitored in 3D hydrogels in the presence of varying biological cues including lung cancer cell secretome. HUVEC and HULEC-5a cells demonstrated comparable growth and morphology on 2D. However, in 3D lung-derived ECM hydrogels, tissue-specific HULEC-5a cells exhibited much better adaptation to their microenvironment, characterized by enhanced organotypic branching and longer branches. HULEC-5a growth was responsive to lung cancer cell-conditioned medium in both 2D and 3D conditions. In 3D, the concentration of ECM ligand significantly affected cell growth in long-term culture where molecular crowding had an inhibitory role. Our data reveals that HULEC-5a cells offer a reliable alternative to frequently pursued HUVECs with comparable growth and morphology. Due to their intrinsic program for cellular crosstalk with resident cells, the use of tissue-specific endothelium constitutes a vital aspect for modeling physiological and pathological processes. Furthermore, our study is the first demonstration of the synergy between lung-specific microvasculature with lung-specific ECM within a 3D *in vitro* model.

Keywords: *Cancer; endothelial cells; extracellular matrix; lung microenvironment; tissue engineering*

1. Introduction

Endothelial cells form an intact and continuous lining which constitute veins, capillaries, arterioles, heart, and lymphatic vessels (Bloom, 2023). They demonstrate high levels of cellular plasticity as they adopt distinct functions within different tissues and organs of the body. The endothelium acts an integral active machinery which supports tissues via enabling nutrient and oxygen flow, mediates the fluidity of the blood, and serves as an immune regulator (Hennigs, 2021). On the other hand, tissue-specific endothelium has the ability to secrete specialized factors which regulate developmental processes, regeneration, physiological homeostasis, and stem-cell activation. Moreover, resident non-endothelial cells in tissues

* Corresponding author.
E-mail address: ozturkece@ku.edu.tr (E. Ozturk).
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similarly secrete factors to stimulate endothelial cells which supports the formation of tissue-specific micro-vasculature (Barabutis et al., 2016; Rafii et al., 2016). Therefore, this reciprocal crosstalk is crucial for the proper functioning of distinct tissues and organs. Human umbilical vein endothelial cells (HUVECs) are one of the most prominent and highly demanded primary endothelial cell source in the field due to their expression of generic endothelial markers, easy isolation, and culturing processes. HUVECs have been widely used in literature to reveal key information regarding the biology of endothelium including colony-stimulating activity, vessel permeability, and established as a model system to study angiogenesis as well as pathological phenomena including atherosclerosis, and inflammation (Han and Geng, 2011; Jang et al., 2017; Medina-Leyte et al., 2020; Tatla et al., 2021). Although non-specific endothelial cells such as HUVECs have dominated the field, they are limited by the lack of representing the high variety of tissue-specific endothelial phenotypes which support tissue and organ functioning (Jourde-Chiche et al., 2019; Rosen et al., 2023). Thus, implementation of tissue-specific endothelium is needed for increasing the validity and accuracy of experimental models (Urbanczyk et al., 2022; Wakabayashi and Naito, 2023).

Lung-specific endothelium is more than just a simple barrier and holds vital importance in the proper functioning of the lung. It resides within lung-specific extracellular matrix (ECM), undergoes continuous stretch and encounters foreign materials during respiration. Under these external stresses, lung endothelial cells form a tightly regulated dynamic sheet. Any interference with this microenvironmental regulation contributes to pathological conditions such as chronic obstructive pulmonary disease (COPD), pneumonia, and hypertension (Goncharova et al., 2020). In cancer, endothelial cells are a vital part of the tumor microenvironment which through neo-angiogenesis, support tumor growth and dissemination. Cancer cells modulate the behavior of tissue-specific endothelium and promote their chaperoning of malignant progress (Hida et al., 2018; Klein, 2018). Therefore, cellular crosstalk between endothelial and tumor cells is a critical regulator of disease.

Extracellular tissue microenvironment is another essential impact on the heterogeneity and specification of endothelium. Biochemical content of ECM as well as mechanical cues act as key regulators of endothelial cell fate and behavior (Nguyen, 2021). Three-dimensional culture models which enable interaction of cells with ECM cues offer biomimetic approaches for studying cellular behavior. Decellularization of native organs is a promising strategy which supports preservation of tissue-specific ECM and reconstitution into hydrogels that can be used as 3D scaffolds (Kusoglu et al., 2023).

Physiologically relevant lung tissue models which employ tissue-specific endothelium are needed for a better understanding of the role of endothelium in homeostatic and pathological conditions. HULEC-5a, as a lung microvascular cell line, contains various features of the primary vascular cells, yet studies on the characterization of this cell line are very limited. Unlike HUVECs, these cells are insufficiently characterized in terms of culture conditions, growth medium requirements, and 3D microenvironmental adaptability. In this study, we aimed to thoroughly characterize lung-specific endothelium in conventional 2D as well as in 3D hydrogels derived from decellularization of native lung tissues. Our results demonstrate the potential use of lung-specific endothelium in vascularized lung tissue models and provide a promising tool for developing therapeutic approaches in lung-related vascular diseases.

2. Materials and methods

2.1. 2D cell culture

The human primary umbilical vein endothelial cells, HUVEC (PCS-100-010™), immortalized human lung microvascular endothelial cells, HULEC-5a (CRL-3244™) were purchased from American Type Culture Collection (ATCC). In all experiments, endothelial cells were used between passage (p) 1 and 4 and were incubated in an environment of 37°C and 5% CO_2. Cells were either cultured in Endothelial Cell Growth Basal Medium-2 (EBM™-2 CC-3156) supplemented with EGM™-2 SingleQuots™ supplement kit (Lonza, CC-4176), endothelial culture medium (EC)-1, EC-2, EC-3, PneumaCult™ ExPlus Medium (StemCell Technologies), Wnt-conditioned medium (Wnt-CM) or A549-CM. Serum-free generic EC medium recipe is formulated with DMEM/F12 as basal medium supplemented with 0.25 mg/ml BSA, 10 ng/ml VEGF and FGF, 0.1% ITS, 2% L-glutamine, 2% Pen-strep, 0.2 µg/ml hydrocortisone, 100 µg/ml ascorbic acid. Then, specific EC-1, EC-2, and EC-3 mediums were prepared by adding 50 µg/ml bovine pituitary extract (BPE), insulin-like growth factor (IGF-1), and T3 (3,3′,5-triiodo-L-thyronine sodium salt) respectively to the generic serum-free EC medium. For the Wnt-CM, 1×10^6 L-Wnt3A cells were cultured in DMEM/F12 basal medium supplemented with 10% fetal bovine serum (FBS) and 0.4 mg/ml geneticin for 4 days. First batch of media was collected and filter-sterilized. 12 ml fresh culture media was added, and cells were cultured until they were confluent. Second batch of media was collected, filter-sterilized, and the two batches were mixed with 1:1 ratio. Next, this conditioned medium is mixed with EGM-2 at a proportion of 1:1 to formulate Wnt-CM. For A549-CM, 1×10^6 A549 cells were cultured for 4 days, and their media was collected and filter-sterilized. Similarly, collected media was mixed with EGM-2 at a proportion of 1:1.

2.2. MTT assay

5 mg MTT (Invitrogen™, M6494) was dissolved in 1 ml of Phosphate Buffered Saline (PBS) and filter-sterilized. Old culture media was aspired, and a fresh medium-MTT mixture (100 µl medium and 10 µl MTT) was added to the wells of a 96-well plate containing various cell concentrations. 4 hours of incubation at 37°C was implemented. The precipitate was dissolved in 100 µl DMSO, incubated at 37°C for 10 minutes which was followed by 5 minutes of shaking. Absorbance values were read at 570 nm with a plate reader. Three replicates were used.

2.3. CTG (Cell Titer Glo) 2D assay

HUVEC and HULEC-5a cells were cultured in a 96-well plate with EGM-2**.** 10 µl CTG reagent (Promega) was added into 100 µl medium (1:10). The lysis procedure was implemented via 2 minutes of orbital shaking. Luminescence values for three replicates were recorded upon 10 min room temperature

stabilization of the plate.

2.4. Decellularization of lung tissue

Fresh bovine lungs were obtained from a local slaughterhouse. Bovine lung tissue was washed thoroughly with ultrapure water supplemented with penicillin/streptomycin (P/S) and immersed in a 2% iodine solution. Then, tissues were subjected to 5 consecutive freeze-thaw cycles of 2 min freezing in liquid nitrogen and 10 min thawing in a 37°C water bath. Next, 10U/ml DNase (in 10 mM $MgCl_2$ buffer, pH:7.5) treatment was performed which was followed by a sterile dH_2O wash for 3 days.

2.5. Pepsin digestion

Decellularized tissue pieces were lyophilized and then cryo-milled into a fine powder for further digestion procedures. Digestion was performed with incubation of samples within 1 mg/ml pepsin in 0.01M HCl solution at room temperature under constant stirring for 48 hours. ECM concentration of 15 mg/ml and 20 mg/ml was used for digestion. Digested ECM samples were then neutralized to physiological conditions (pH 7, 1X PBS) by adding NaOH and 10X PBS. These pre-gel forms were stored at -20°C for further studies.

2.6. 3D cell culture

For encapsulation into 3D hydrogels, cell suspensions were gently mixed with cold pre-gel dECM solutions at a concentration of 2×10^6 cells/ml. The mixture was then cast onto well plates, and incubated at 37°C for 45 minutes until proper gelation after which culture medium was carefully added. Hydrogels were incubated under 37°C and 5% CO_2 conditions and the culture medium was changed every other day until the end of the experiment.

2.7. 3D CTG assay

HUVEC and HULEC-5a cells that were embedded in decellularized ECM (dECM) gels in a 24-well plate were cultured and monitored for 4 weeks. CellTiter-Glo 3D (Promega) assay was utilized on days 1, 14, 21 and 28. After 1 hour of CTG incubation, culture media was collected and transferred into a 96-well plate where luminescence was measured with a microplate reader. A growth curve was generated for each condition tested.

2.8. Phalloidin/DAPI staining

Cells which were seeded on glass-bottom 24-well plates were first fixed with 4% PFA for 15 min and then washed three times with PBS. Wells were incubated with a blocking solution containing PBS and 5% normal goat serum for 1 hour. Cells were incubated with phalloidin solution (Phalloidin-iFluor 647 Reagent, ab176759) for 45 min and then incubated with DAPI for 5 min in the dark at room temperature. Imaging was performed using a Leica SP8 confocal microscope.

2.9. Statistics

Statistical analyses were performed using Prism 9 (GraphPad). Data were analyzed using an unpaired t-test when two independent groups were compared (minimum n=3). The results are expressed as mean and error with a 95% confidence interval. A p-value$<$0.05 was considered statistically significant.

3. Results and discussion

This study aimed to implement a thorough characterization of lung-specific endothelial cells in terms of growth kinetics, morphological features, response to biochemical stimuli as well as their interaction with 3D native lung-derived matrices (Fig. 1). The first part of the study focused on the assessment of cell growth and behavior on 2D conventional cultures to compare tissue-specific HULEC-5a cells to the widely adopted HUVEC cells. Our results demonstrate that both cell types revealed similar actin cytoskeletal structure and elongated cellular morphology (Fig. 2A). On the other hand, HULEC-5a cells exhibited significantly smaller cell size (Fig. 2B). Differences in cell size could be attributed to the fact that HULEC-5a is a microvascular endothelial cell type whereas HUVEC is vein-derived. Cells lining larger vessels like veins have been reported to have relatively larger size (Kruger-Genge et al., 2019). We then assessed the growth kinetics of HUVEC and HULEC-5a cells. Both cell types were seeded at a constant density of 20×10^3 cells per each well of a 96-well plate and growth was monitored over 3 days in culture (Fig. 2C). The results indicated that lung-specific and generic endothelial cells did not reveal any significant difference in proliferation rate. Then we investigated the effect of initial cell seeding density on the growth dynamics of HULEC-5a cells up to a week (Fig. 2D). The growth curves demonstrated that seeding cells at a density of 500×10^3 cells per well of a 96-well plate caused an immediate drop in cell growth which then was stabilized. A density of 100×10^3 cells/well on the other hand did not promote further cell growth but maintained the initial conditions. The optimal cell seeding density for obtaining a proper growth curve from HULEC-5a cells was in the range of 10-50 $\times 10^3$ cells per well.

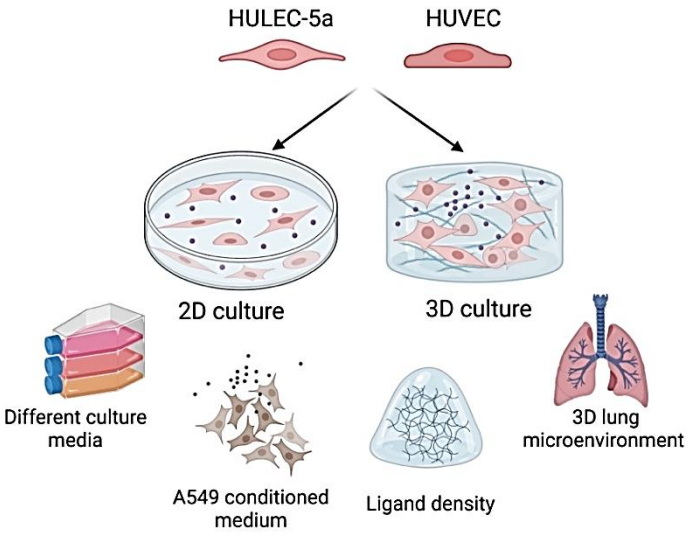

Fig. 1. Schematic description of the study (Created with BioRender).

Next, we assessed the effects of different growth medium formulations on lung-specific endothelial cells in terms of cellular morphology and growth dynamics (Fig. 3). We used a commercially available culture medium optimized for endo-

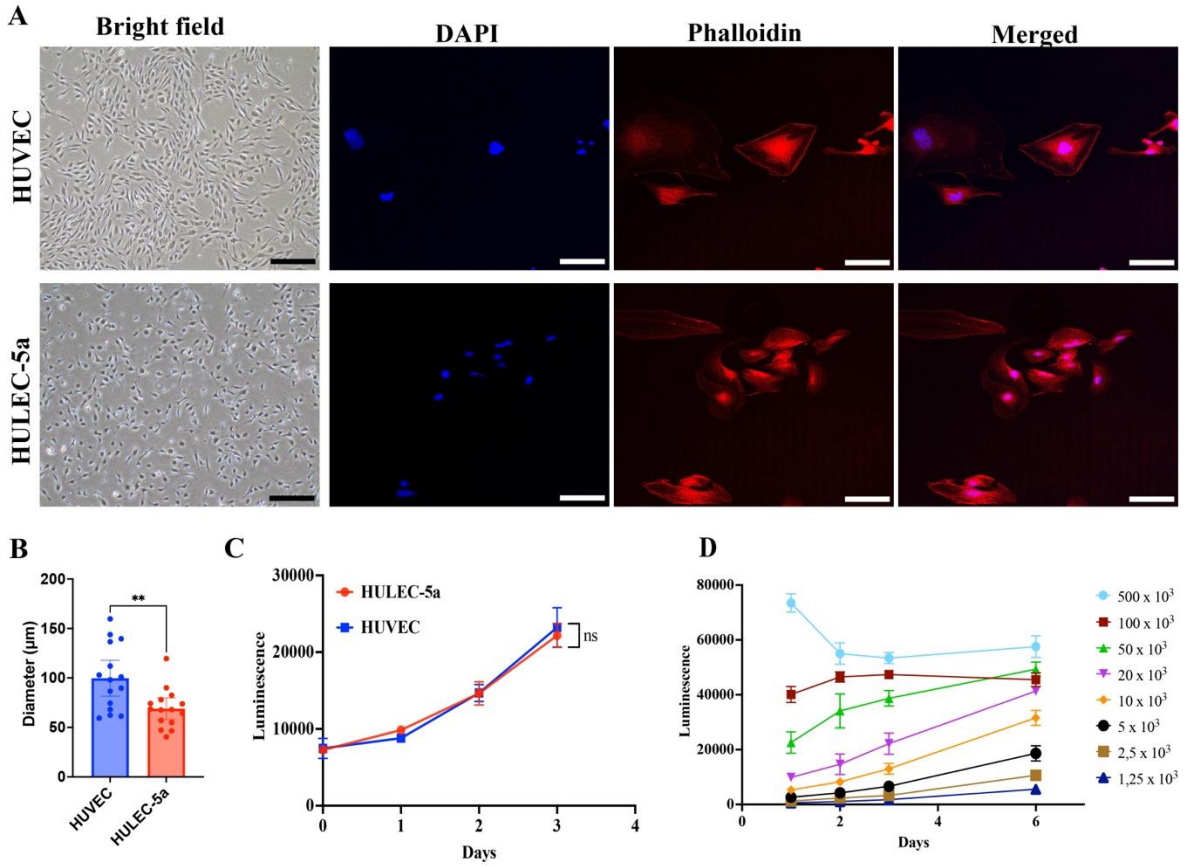

Fig. 2. Comparison of HUVEC and HULEC-5a growth and morphology on 2D cultures. (A) Phenotypic characterizations of endothelial cells: Bright field images (Scale bars: 150 µm) and confocal microscopy images for phalloidin-DAPI staining (Scale bars: 30 µm). (B) Diameter size differences of HUVEC and HULEC-5a under 20X magnification. (C) Growth curves of HUVEC and HULEC-5a for 3 days on 2D culture. (D) Growth curves of HULEC-5a with differing initial cell seeding density on 2D culture.

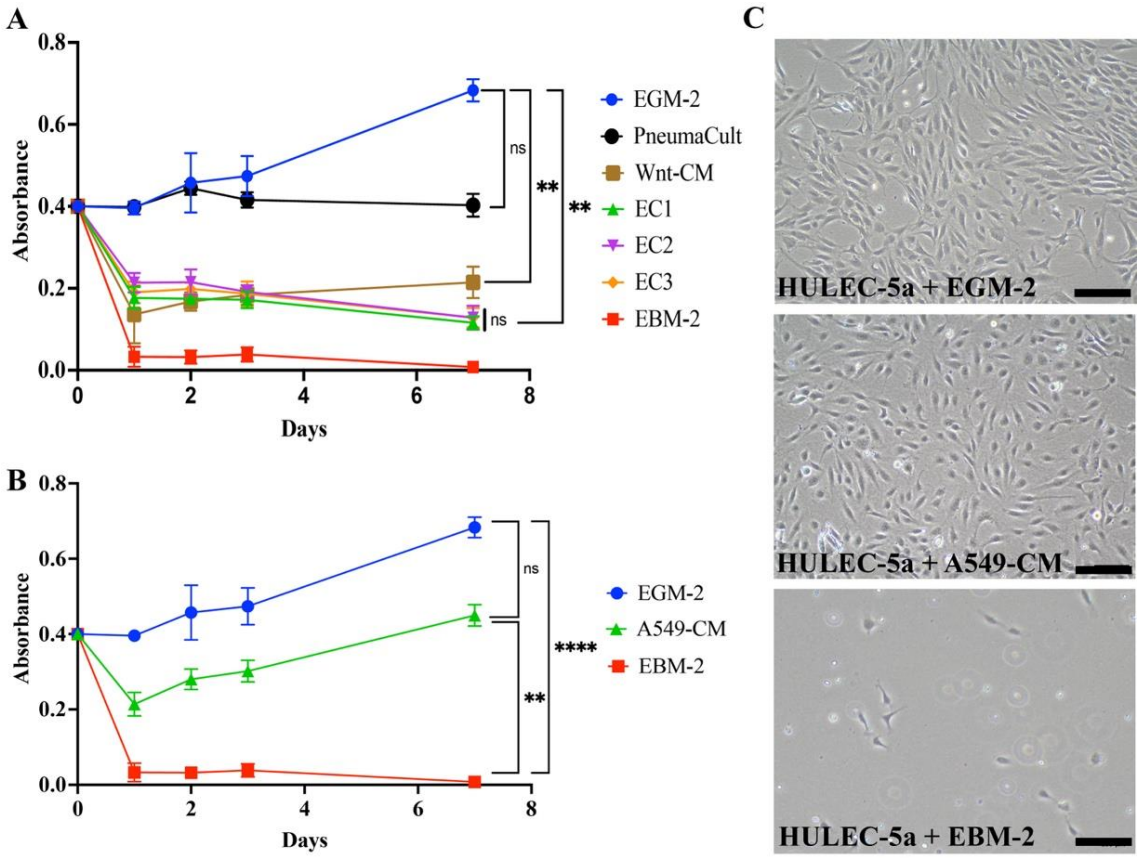

Fig. 3. HULEC-5a growth on 2D. (A) Growth of HULEC-5a cells within different medium conditions and (B) in A549-conditioned medium assessed by MTT assay. (C) Bright field images of HULEC-5a cells cultured in EGM-2, A549-CM and EBM-2 media. (Scale bars: 100 µm).

thelial cells, EGM-2, as a positive control, whereas basal medium without endothelium-supporting supplements was used as a negative control (EBM-2). Moreover, we monitored HULEC-5a cells in different media formulations to find out lung-specific endothelial cell synergy with media content. Culture media compositions with the ability to support different cell types within engineered tissues are a critical challenge in the field. Vascularized, engineered tissue models combine endothelial cells with tissue-resident epithelial and stromal cells, and all those cell types have different requirements of biological cues for growth and functioning. Therefore, it is crucial to find formulations that provide an optimal media to support collective cell growth in such co-culture models. Accordingly, to assess the potential use of HULEC-5a cells in vascularized *in vitro* lung tissue models, we tested their growth in media optimized for pulmonary epithelium such as PneumaCult™ (Rayner et al., 2019). HULEC-5a growth in PneumaCult™ was comparable to EGM-2 for the first three days of culture, however, after 7 days, cell growth in EGM-2 was significantly higher whereas cells ceased to grow in PneumaCult™ (Fig. 3A). This indicates that using growth media formulated for pulmonary epithelium could only serve as an approach for short-term co-cultures whereas lack of endothelium-specific cues hinders their use for long-term cultures of vascularized *in vitro* lung models. Next, we wanted to examine the effect of several serum-free media formulations on lung-specific endothelial cell growth. Undefined composition and batch-to-batch variations in sera have motivated the development of more defined, serum-free formulations for endothelial cell culture (Andrée et al., 2019). We have adapted a serum-free formulation (EC) which has been reported to support the growth of HUVEC cells in the literature. However, the majority of studies that employed this serum-free approach demonstrated complementary techniques such as cultivating endothelial cells with stromal cells to induce growth with the aid of paracrine factors (Huttala et al., 2015). Since the use of stromal cells may also lead to experimental variability, we aimed to avoid their use and opted to enrich the EC formulation with additional supplements. EC medium was supplemented with BPE (EC-1), IGF-1 (EC-2) and T3 (EC-3) to generate three distinct defined media formulations. BPE, IGF-1, and T3 have been shown to have additional and dose-dependent growth-stimulating effects on endothelial cells which was specifically established for microvascular endothelium (Balzan et al., 2013; Lin et al., 2017). Similarly, we assessed HULEC-5a growth in all three formulations for 7 days (Fig. 3A). However, none of the three media supported the growth of lung-specific endothelium. Next, we examined the effect of Wnt-CM, derived from the culture of L-Wnt3A cells, on the growth of HULEC-5a cells. Wnt signaling has been established as an important regulator of endothelial cell growth and functioning. Being an evolutionarily conserved pathway with crucial roles in embryonic development, Wnt signaling has been shown to have a key importance in cell fate determination. It has been reported to have a role in survival, proliferation as well as plasticity and specification of endothelium (Dejana and Kuhl, 2010). Therefore, we tested Wnt-CM as a means to promote the growth of lung-specific endothelial cells. Contrarily, Wnt-CM had an adverse effect on both proliferation and morphology of HULEC-5a cells (Fig. 3A). Cancer cell secretome is another crucial regulator of endothelial cell growth and heterogeneity (McHenry and Prosperi, 2023). Cancer progression and metastasis heavily depend on the angiogenic switch which involves neo-vascularization. Therefore, cancer cells and endothelial cells affect each other with continuous reciprocal communication. Cancer cells specifically activate the proliferation of endothelial cells that are present in neighboring tissues and promote their specialization. As a result, a unique tumor-associated endothelium is created by the cancer cells, constitutively promoting their growth and dissemination (Ritchie et al., 2021; Zahari et al., 2023). Thus, we next aimed to inspect the effect of cancer cell secretome on the growth kinetics of HULEC-5a cells. Conditioned medium was collected from the cultures of a non-small cell lung adenocarcinoma cell line (A549-CM). As anticipated, A549-CM strongly promoted the growth of HULEC-5a cells (Fig. 3B). Even though the stimulation of cell growth was not to the extent achieved with EGM-2, the growth curves of both media formulations demonstrated a similar trend in which endothelial cells still preserved their proliferative abilities after a week of culturing. As opposed to other media formulations (EBM-2, Pneumacult™, EC-1, EC-2, EC-3, Wnt-CM), A549-CM supported the maintenance of growth and distinct endothelial cell morphology (Fig. 3C). Therefore, our data confirms that lung cancer secretome has growth-stimulatory effects on lung-specific microvasculature. Such synergy between lung cancer cells and lung endothelium supports the development of *in vitro* vascularized tumor tissue models which allow investigation of the role of cellular interactions in disease progression.

After characterizing the growth kinetics of HULEC-5a in comparison to HUVEC cells and response to different media formulations with distinct biological cues, we assessed the use of HULEC-5a cells within a more physiologically relevant, 3D tissue model (Fig. 4). Conventional 2D cultures lack a faithful representation of the intricate architecture of native tissue microenvironments where cell-cell and cell-matrix interactions play a vital role in regulation of biological processes in both homeostasis and disease conditions. 3D cultures on the other hand, offer physiological relevance through mimicking the key biochemical and physical aspects of the ECM (Mierke, 2023). 3D culturing has been shown to have an effect on the growth, differentiation and phenotypic stability of endothelial cells as well as vascular network formation (Paek et al., 2019). Native ECM-derived hydrogels are frequently pursued in tissue engineering for their ability to present tissue-specific extracellular cues. They are comprised of decellularization of native organs and reconstitution into hydrogels within which cell types of interest can be embedded for modeling a plethora of cellular processes. This allows the preservation of tissue-specific ECM cues and the investigation of cell-matrix interactions. We fabricated bovine lung-derived ECM hydrogels (dLung) to assess the growth and behavior of lung-specific microvasculature within a native-like microenvironment (Fig. 4A). HULEC-5a and HUVEC cells were encapsulated in dLung gels and cultured for 10 days to compare their growth and organotypic branching ability in 3D. Both cell types exhibited adaptation and branching patterns in dLung hydrogels (Fig. 4B). Further morphological assessment was done with quantification of branch number and branch length. Interestingly, lung-specific microvasculature in lung ECM-derived hydrogels demonstrated a significantly higher number of branches with increased length compared to non-specific HUVEC cells (Fig. 4C). This indicates a synergy between tissue-specific endothelium with tissue-specific ECM. Such synergy and microenvironmental adaptation are critical for promoting endothelial cells' ability to curve the surrounding matrix for further sprouting and angiogenic events in different physiological processes.

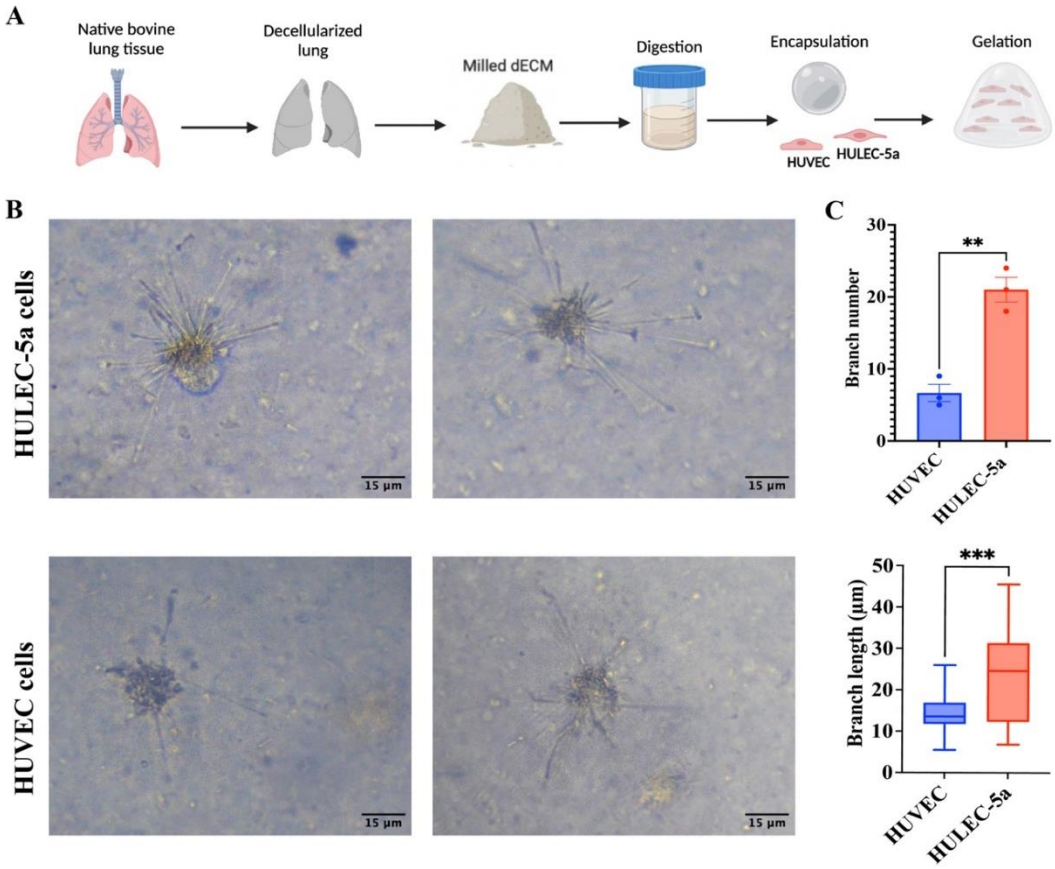

Fig. 4. 3D culturing of endothelial cells within decellularized lung-derived ECM hydrogels. (A) Scheme depicting the decellularization procedure for bovine lungs. (B) Encapsulation of HULEC-5a and HUVEC into dLung (15 mg/ml) hydrogels (Scale bar: 15µm). (C) Quantification of branch number and length of HULEC-5a and HUVEC in dLung hydrogels.

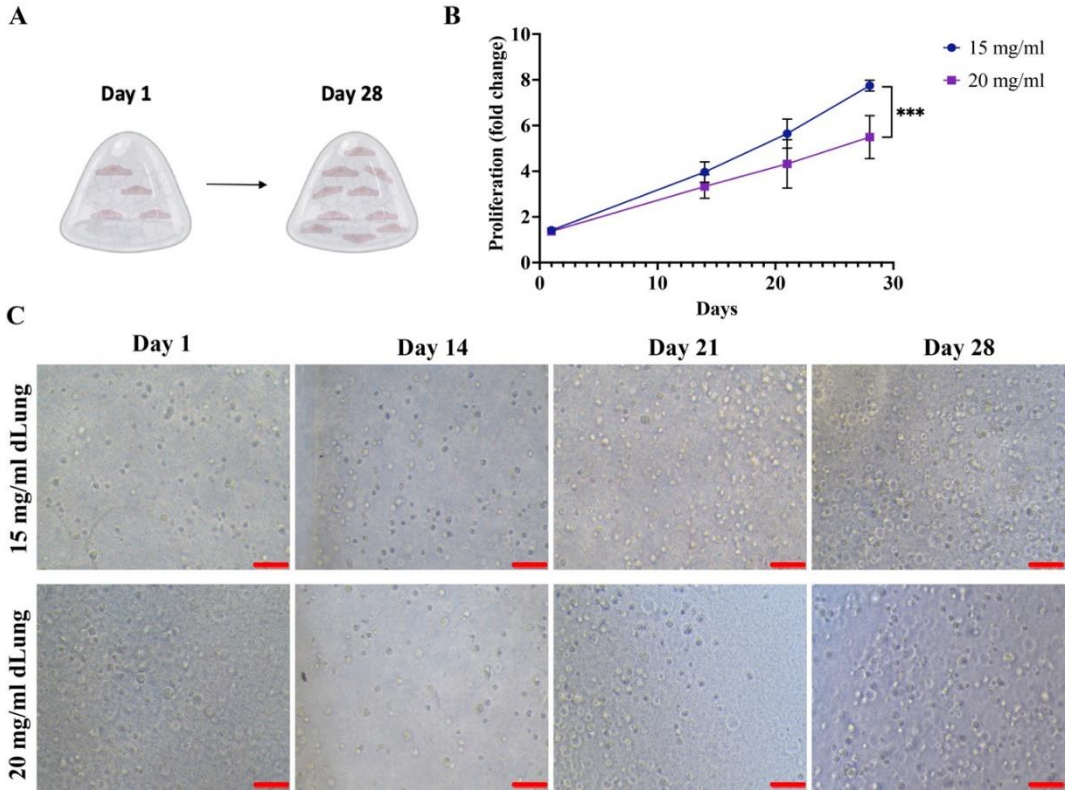

Fig. 5. Encapsulation and long-term growth monitoring of HULEC-5a in dLung hydrogels with changing ligand density. (A) Scheme representing encapsulation and 3D culturing of endothelium. (B) Cellular proliferation of HULEC-5a within dLung hydrogels with 15 mg/ml or 20 mg/ml ECM ligand density up to 28 days assessed with CTG assay and normalized to day 0. (C) Bright field images of HULEC-5a cells in dLung gels on days 1, 14, 21 and 28 (Scale bar: 70 µm).

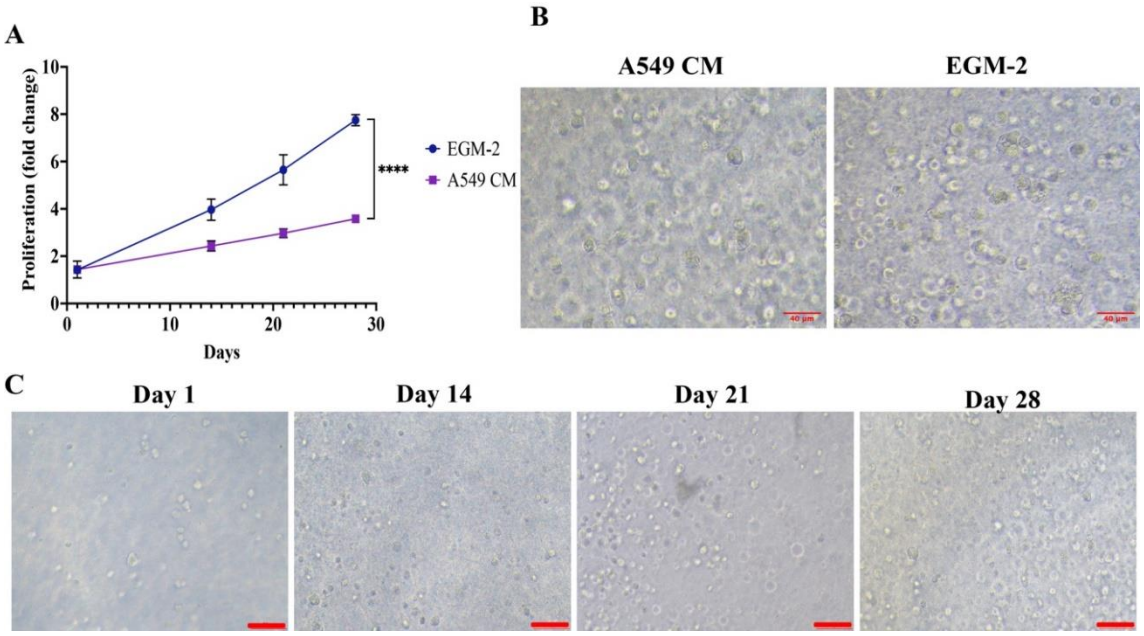

Fig. 6. The effect of lung cancer cell secretome on the growth of HULEC-5a in dLung hydrogels. (A) Cellular proliferation of HULEC-5a within dLung hydrogels in the presence of EGM-2 or A549-CM up to 28 days. (B) Brightfield images of HULEC-5a cells in dLung hydrogels within EGM-2 or A549-CM. (Scale bar: 40 µm). (C) Brightfield images of HULEC-5a cells in dLung gels within A549-CM on days 1, 14, 21 and 28. (Scale bar: 70 µm).

We then evaluated the growth kinetics of HULEC-5a cells within dLung hydrogels over 4 weeks (Fig. 5A). Additionally, we assessed the effect of ECM ligands via modulating the native matrix concentration in the hydrogels. dLung hydrogels supported the long-term culturing of HULEC-5a cells and stimulated their growth at both 15 mg/ml and 20 mg/ml ECM concentrations (Fig. 5B and 5C). Cellular growth was comparable in both hydrogels for 3 weeks, however, 15 mg/ml hydrogels yielded a significantly higher number of cells at the end of 4 weeks (Fig. 5B). This reveals that ECM crowding had a particularly adverse effect on the growth of endothelium. Furthermore, an increase in ligand content alters the mechanical properties of the hydrogels and correlates with increased stiffness. Tissue stiffness is a known regulator of endothelial cell growth and behavior which could account for the differences observed within the two hydrogel fields (Gordon et al., 2020).

We next looked into the effect of A549-CM on HULEC-5a cells within 3D, native ECM-derived, dLung hydrogels (Fig. 6). As opposed to 2D, dLung hydrogels provide a more representative model for the lung tissue microenvironment. It has been established in the literature that cellular behavior on 2D marked by adherent morphology is quite different than in the 3D (Jensen and Teng, 2020). Nonetheless, cancer research has heavily relied on conventional 2D cultures and the intersection of tissue engineering and cancer fields has been rather recent. Various important oncogenic signaling routes are altered in 3D, as key molecular players get either inactive or hyperactive in 3D, such as YAP (Lee et al., 2019) and ROCK (Matsubara and Bissell, 2016). Similarly, the expression pattern of pro-angiogenic factors on both transcriptional and translational levels is altered within the 3D context (Fontana et al., 2020). Therefore, we aimed to investigate the growth and morphological changes in lung-specific endothelium in response to biological cues presented by the cancer cells within a physio-mimetic, 3D context. A549-CM promoted the growth of HULEC-5a in dLung hydrogels although to a lesser extent when compared with EGM-2 (Fig. 6A). Different media formulations did not have an observable effect on cellular morphology (Fig. 6B). On the other hand, long-term culturing of HULEC-5a up to 28 days in dLung hydrogels within A549-CM conditions (Fig. 6C) and maintenance of cellular growth provides a promising *in vitro* model which enables the use of lung-specific endothelium within native lung matrix and allows investigation of the molecular effects of cancer cell secretome on microvasculature.

4. Conclusion

In conclusion, our experimental data demonstrates that HULEC-5a cells provide a lung tissue-specific endothelial cell source with growth kinetics and morphological characteristics comparable to the widely used HUVEC cells. The study also denotes the proliferative impact of the lung cancer secretome on microvascular lung endothelial cells that can preserve the proper morphology of HULEC-5a cells. Furthermore, native lung-derived ECM hydrogels provide a physio-mimetic microenvironment for endothelium and support their organotypic branching, a phenomenon not observed in conventional 2D culturing. To our knowledge, this is the first study that investigated endothelial cell behavior in native lung-derived 3D hydrogels. The better adaptation of HULEC-5a cells to dLung hydrogels in terms of the number of branches and branch length compared to HUVECs emphasizes the importance of models that enable investigation of tissue-specific cell types within relevant matrices. Furthermore, such models allow investigation of particular molecular effects, such as paracrine signaling in the tumor microenvironment, and have the potential to shed light on the complex cellular crosstalk between cancer cells and endothelium.

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Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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